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Characterization of the Interactions between Calmodulin and Death Receptor 5 in Triple-negative and Estrogen Receptor-positive Breast Cancer Cells

AN INTEGRATED EXPERIMENTAL AND COMPUTATIONAL STUDY[*][S]

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Activation of death receptor-5 (DR5) leads to the formation of death inducing signaling complex (DISC) for apoptotic signaling. Targeting DR5 to induce breast cancer apoptosis is a promising strategy to circumvent drug resistance and present a target for breast cancer treatment. Calmodulin (CaM) has been shown to regulate DR5-mediated apoptotic signaling, however, its mechanism remains unknown. In this study, we characterized CaM and DR5 interactions in breast cancer cells with integrated experimental and computational approaches. Results show that CaM directly binds to DR5 in a calcium dependent manner in breast cancer cells. The direct interaction of CaM with DR5 is localized at DR5 death domain. We have predicted and verified the CaM-binding site in DR5 being ^{354}WEPLMRKLGL^{363} that is located at the α2 helix and the loop between α2 helix and α3 helix of DR5 DD. The residues of Trp-354, Arg-359, Glu-355, Leu-363, and Glu-367 in DR5 death domain that are important for DR5 recruitment of FADD and caspase-8 for DISC formation to signal apoptosis also play an important role for CaM-DR5 binding. The changed electrostatic potential distribution in the CaM-binding site in DR5 DD by the point mutations of W354A, E355K, R359A, L363N, or E367K in DR5 DD could directly contribute to the experimentally observed decreased CaM-DR5 binding by the point mutations of the key residues in DR5 DD. Results from this study provide a key step for the further investigation of the role of CaM-DR5 binding in DR5-mediated DISC formation for apoptosis in breast cancer cells.

Breast cancer is one of the most common malignancies among American women and affects an estimated 1.3 million women annually worldwide (1, 2). Chemotherapy has steadily increased breast cancer survival rate; however, drug resistance and systemic toxicity remain critical issues that hinder effective breast cancer treatment (3–7). Better understanding of the pathogenesis of breast cancer could facilitate the development of novel strategies for targeted therapy for effective breast cancer treatment. Classification of breast cancers into receptor-based subtypes, including estrogen receptor (ER)[3]-positive or triple-negative breast cancers, establish targets that guide individualized treatment for patients (8). Drug-resistant ER-positive breast cancers and triple-negative breast cancers are prolific breast cancers with poor clinical outcome (3). Death receptor-5 (DR5) is expressed in various breast cancer cell lines that represent the different breast cancer subtypes (9, 10), and DR5 expression is up-regulated in breast cancer cells compared with normal breast tissue (11).

DR5, also called tumor necrosis factor (TNF)-related apoptosis inducing ligand receptor 2 (TRAIL-R2), is a member of the TNF receptor superfamily and is one of five receptors bound by TNF receptor apoptosis-inducing ligand (TRAIL). TRAIL has been reported with strong antitumor activity (12–14), however, TRAIL can induce apoptosis of normal human hepatocytes (15). TRA-8 is a DR5-specific agonist antibody and TRA-8 activation of DR5 has shown tumoricidal activity *in vitro* and *in vivo* without inducing normal hepatocyte apoptosis (16). TRA-8 can induce DR5-mediated apoptosis in breast cancer cells (9, 17).

DR5 contains a cytoplasmic death domain (12) and transduces its apoptotic signal via death-inducing signaling complex (DISC) formation and activation of caspase signaling (18). Upon stimulation by binding of TRA-8 to its extracellular domains, DR5 oligomerizes into homotrimers (19). DR5 homotrimers are bound at their death domains (DD) by the adapter protein Fas-associated death domain protein (FADD). FADD recruits procaspase-8 forming DISC leading to caspase-8 activation (18, 20–22). Dysfunction of the DR5 DD or DISC components can lead to resistance of DR5-mediated apoptosis (18, 22). DR5 DD mutations have been identified in breast cancer (23). Studies show that some key residue mutations in DR5 DD result in DR5 unable to recruit FADD and

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[3] The abbreviations used are: ER, estrogen receptor; CaM, calmodulin; DISC, death-inducing signaling complex; DR5, death receptor 5; DD, death domain; CR, cytoplasmic region; BSD, binding site deletion; FADD, Fas-associated death domain protein; TRAIL, TNF receptor apoptosis-inducing ligand.

caspase-8 for DISC formation to signal apoptosis (24–26). Leu-363 is the residue corresponding to the site of *lpr* mutation in the mouse Fas receptor, and L363N, L363A, and L363F (mutation related to non-small cell lung cancer (27)) mutations in DR5 DD result in the failure of inducing apoptosis. Arg-359 is the conserved residue among DR5, DR4, DR3, TNFR1, and Fas, and DR5 R359A mutant can completely lose the function of DR5. Glu-355 has been identified in human tumors, and DR5 E355K mutant (mutation related to non-small cell lung cancer and gastric cancer (27, 28)) results in the DR5 losing its ability to form DISC. Trp-354 has been identified as an important hydrophobic residue for the overall structure of DR5, and DR5 W354A mutant (*i.e.*W325A in Ref. 24) causes reduced ability of DR5 to induce apoptosis. Glu-367 has been identified in human tumor, and DR5 E367K mutant (*i.e.* E338K in Ref. 25) results in the DR5 losing its ability to form DISC.

Calmodulin (CaM) functions as an intracellular mediator of Ca^{2+} signals and regulates various cellular processes (29, 30). Breast tumor transformation to malignancy is associated with the increase in CaM expression (31, 32). CaM binding to Fas, a member of the TNF receptor superfamily, has been well characterized (33–37). CaM binds to Fas at the Fas death domain (34) and CaM is recruited into the Fas-mediated DISC after Fas activation in cholangiocarcinoma cells (33). A role of CaM in DR5-mediated apoptotic signaling regulation has been reported in human lung cancer cells (38) and hepatocellular carcinoma cells (39). The mechanism of CaM regulation of DR5-mediated apoptotic signaling has not been fully understood. The structure of CaM consists of 8 helices, forming four calcium-binding loops, which exhibit the characteristic EF hand structure (40). The dumbbell-shaped structure of CaM is composed of two globular terminal regions and two helices comprising the inter-domain region (41). CaM interacts with diverse proteins to regulate cellular activities in a Ca^{2+}-dependent manner (34, 42, 43). Three main classes of recognition motifs exist for many of the known CaM-binding proteins (34, 45): [1] Ca^{2+}-dependent binding 1–8-14 motif ($[FILVW]_1XXXXXX[FAILVW]_8XXXXX[FILVW]_{14}$), [2] Ca^{2+}-dependent binding 1–10 motif ($XXX[FILVW]_1XXXXXXXX[FILVW]_{10}$), in which the fifth residue is usually also a conserved residue, *i.e.* 1–5-10 motif ($XXX[FILVW]_1XXX[FAILVW]_5XXXX[FILVW]_{10}$), and [3] Ca^{2+}-independent binding IQ motif ([ILV]Q*XXX*R*XXXX*[RK]*XX*[FILVWY]).

In this study, we characterized the Ca^{2+}-dependent direct interactions between CaM and DR5 in estrogen receptor (ER)-positive ZR-75-1 and triple-negative MDA-MB-231 breast cancer cells. We identified the CaM-binding site in the DR5 death domain and the critical residues in the DR5 death domain for CaM-DR5 binding using integrated computational and experimental approaches.

Experimental Procedures

We characterized the direct interactions between CaM and DR5 in ER-positive ZR-75-1 and triple-negative MDA-MB-231 breast cancer cells using complex-immunoprecipitation (Co-IP) and CaM pull-down assay. We determined that CaM directly binds to the purified recombinant DR5 death domain using CaM pull-down assays. We identified the CaM-binding site in the DR5 death domain and the critical residues in the DR5 death domain for CaM-DR5 binding using integrated computational and experimental approaches.

Breast Cancer Cell Culture—ER-positive ZR-75-1 and triple-negative MDA-MB-231 breast cancer cells were kindly provided by Dr. Donald Buchsbaum or Dr. Tong Zhou at the University of Alabama at Birmingham. ZR-75-1 cells were cultured in HAM's K-12 medium with 20% FBS. Triple-negative MDA-MB-231 breast cancer cells were cultured in DMEM medium (Thermo Fisher Scientific, Waltham, MA) with 10% FBS and supplemented with MEM vitamins, nonessential amino acids, sodium pyruvate, and L-glutamine. Both cell lines were maintained in the medium with 10% FBS, supplemented with 1% penicillin, 1% streptomycin, and 1% amphotericin B antibiotic at 37 °C, 5% CO_2, and 95% relative humidity atmosphere.

Prediction of CaM-binding Site on DR5 Death Domain (DR5 DD) using Computational Methods—The CaM-binding site in DR5 was predicted using the combined text pattern search method (46) that was based on the three CaM-binding motifs (34, 45), the hydropathic analysis method (47–53), and the experimentally known residues in DR5 DD critical for DR5-mediated death inducing signaling complex (DISC) formation for apoptosis (24, 25, 27, 54). CaM interacts with diverse proteins to regulate cellular activities in a Ca^{2+}-dependent manner (34, 42, 43). Three main classes of recognition motifs for many of the known CaM-binding proteins (34, 45) include: 1) Ca^{2+}-dependent binding 1–8-14 motif: ($[FILVW]_1XXXXXX[FAILVW]_8XXXXX[FILVW]_{14}$), 2) Ca^{2+}-dependent binding 1–10 motif ($XXX[FILVW]_1XXXXXXXX[FILVW]_{10}$), in which the fifth residue is usually a conserved residue, *i.e.* 1–5-10 motif ($XXX[FILVW]_1XXX[FAILVW]_5XXXX[FILVW]_{10}$), and 3) Ca^{2+}-independent binding IQ motif ([ILV]Q*XXX*R*XXXX*[RK]*XX*[FILVWY]). The sequence of DR5 death domain (DD) was scanned to identify the CaM-binding motif(s) in DR5 DD using text pattern search method (46). To further identify the CaM-binding site in DR5 DD, we adopted complementary hydropathy index sign-matching strategy (55) that was based on the molecular recognition theory of Blalock (56) to predict protein-protein interaction sites. We used the hydropathy binary code (+ and −) of the predicted CaM-binding site(s) in DR5 DD from text pattern search method to match the binary code of hydropathy of CaM and rank the results by the number/percentage of the complementary pairs ((+)-(−)). Together with the consideration of the experimentally known residues in DR5 DD critical for DR5-mediated DISC formation for apoptosis (24, 25, 27, 54), the potential CaM-binding site in DR5 DD was identified and experimentally validated. The text pattern search and complementary hydropathy index sign-matching were performed using the in-house developed codes.

Generation of DNA Constructs of DR5 Fragments Using Polymerase Chain Reaction—Full-length human DR5 cDNA cloned into the pCMV-SPORT6 vector was kindly provided by Dr. John Mountz at the University of Alabama at Birmingham. DR5 cytoplasmic region (DR5 CR) was generated by PCR using pCMV-SPORT6 DR5 as the template. DR5 CR PCR products were cloned into the pET100 vector (Invitrogen Life Technologies, Grand Island, NY) for expression in *Escherichia coli* BL21

(DE3) strain cells. DR5 death domain (DR5 DD), DR5 cytoplasmic region with death domain deletion (DR5 ΔDD), DR5 cytoplasmic region with the predicted CaM-binding site (^{354}WEPLMRKLGL^{363}) deletion (DR5 BSD) were generated by deletion PCR. DR5 cytoplasmic region mutations: DR5 W354A, DR5 E355K, DR5 R359A, DR5 L363N, and DR5 E367K were generated by site mutation PCR using pET100 DR CR as the template. DR5 ΔDD, DR5 BSD, and DR5 cytoplasmic region mutation PCR products with overlapping ends were generated by using a forward primer (5′) together with the reverse primer (3′) coding for the mutation or deletion (supplemental Table S1).

Expression and Purification of Recombinant Proteins—pET100 6×His tag DR5 CR, DR5 DD, DR5 ΔDD, DR5 BSD, and DR5 CR mutations proteins were expressed in *E. coli* BL21 (DE3) strain cells (Invitrogen Life Technologies) as follows. Transformed *E. coli* cells were incubated in LB media with 100 μg/ml ampicillin (Fisher Scientific) on a shaking incubator at 37 °C for 3 h. 1 mM isopropyl β-D-thiogalactopyranoside (Promega) was added to cultures for induction of protein expression and then incubated for an additional 4 h at 37 °C. Recombinant protein expressions were evaluated by Western blot using an anti-6×His antibody (Sigma-Aldrich). After induction of protein expression, *E. coli* cultures were centrifuged and bacteria pellets were lysed by sonication, lysates were centrifuged and the supernatant collected. The 6×His-tagged recombinant proteins were purified by nickel ion affinity chromatography according to the manufacturer's direction for 6×His-tagged protein purification system (Qiagen). Recombinant proteins were further purified by size exclusion chromatography using a HiLoad 16/600 Superdex 75 prepgrade column on an AKTA purifier (GE Life Sciences).

DR5 Immunoprecipitation—To determine an interaction between CaM and DR5 in ER-positive ZR-75-1 and triple-negative MDA-MB-231 breast cancer cells, DR5 was immunoprecipitated (IP) from cell lysates using TRA-8-conjugated Sepharose beads (16), which were kindly provided by Dr. Tong Zhou at the University of Alabama at Birmingham. Iso-IgG-conjugated Sepharose beads (Cell Signaling Technologies, Beverly, MA, specificity: none, source: mouse, Cat. 3420S, Lot 3) were used for a control IP experiment. ER-positive or triple-negative breast cancer cells were lysed using a 50 mM Tris-HCL pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1:100 Halt protease inhibitor (Thermo Fisher Scientific, Waltham, MA) buffer at 4 °C for 30 min. The total protein concentration was determined using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). 5 mg/ml total protein of cell lysates was precleared with 40 μl of 4B-Sepharose beads (Sigma-Aldrich) at 4 °C for 1 h. After the clearance, the supernatants of each sample were incubated with TRA-8-conjugated Sepharose beads or mouse Iso-IgG-conjugated Sepharose beads, and rotated overnight at 4 °C. Following overnight incubation, TRA-8-conjugated Sepharose beads or mouse Iso-IgG conjugated Sepharose beads were washed using a 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100 washing buffer five times. After washing, 40 μl of 2× Laemmli sample buffer with 2-mercaptoethanol was added to the beads to elute the proteins for Western blot analysis.

CaM Pull-down Assay—To determine the calcium-dependent interactions between CaM and DR5 death domain, ER-positive and triple-negative breast cancer cells were lysed with phosphate-buffered saline with 1% Triton X-100 (PBST), and 1:100 Halt protease inhibitor (Thermo Fisher Scientific) supplemented with 1 mM $CaCl_2$ or 2 mM EGTA. Cell lysates were incubated with 100 μl of CaM-4B-Sepharose beads (GE Healthcare Bio-Science) or control 4B-Sepharose beads for 4 h at 4 °C. After CaM pull-down beads or control 4B-Sepharose beads were washed five times with the PBST buffer with 1 mM Ca^{2+} or 2 mM EGTA, and then 100 μl of 2× Laemmli buffer was added to elute proteins for Western blot analysis. To determine the direct interactions between CaM and DR5, purified recombinant DR5 cytoplasmic region (DR5 CR) and DR5 death domain (DR5 DD) proteins were generated. To determine the CaM-binding site in DR5, purified recombinant DR5 CR with death domain deletion (DR5 ΔDD) and DR5 CR with potential binding site deletion (DR5 BSD) proteins were generated. To identify the critical residues in DR5 death domain necessary for CaM-DR5 binding, purified recombinant DR5 CR mutants with the different point mutation were generated. All recombinant DR5 proteins and DR5 mutant proteins were dialyzed into a 50 mM Tris pH 7.6, 120 mM NaCl, 1 mM Ca^{2+}, 1% Brij binding buffer. Recombinant proteins were incubated with 150 μl of CaM-4B-Sepharose beads or control Sepharose beads for 3 h at 4 °C. After pull-down, beads were washed with the 50 mM Tris, pH 7.6, 120 mM NaCl, 1 mM Ca^{2+}, 1% Brij binding buffer, and then 150 μl of 2× Laemmli buffer was added to elute recombinant proteins for Western blot.

Western Blot Analysis—CaM binding to DR5 was assessed by Western blot analysis of eluted CaM or DR5 proteins from IP or CaM pull-downs. Samples from IP and CaM pull-down in Laemmli buffer were heated at 100 °C for 5 min. Protein samples were resolved by SDS-PAGE and transferred onto a 0.45 μm nitrocellulose membrane from Bio-Rad. For IP samples, membranes were incubated overnight at 4 °C with primary antibodies against DR5 (Abcam Cambridge, MA, specificity: DR5 isoform 1 and 2, source: rabbit, Cat. Ab47179, Lot# GR199335–1) and CaM (Millipore Billerica, MA, source mouse, Cat. 05173, Lot 2123697). For samples from CaM pull-down, membranes were incubated overnight at 4 °C with a primary antibody against the 6×His tag (Sigma-Aldrich, specificity: recognizes synthetic polyhistidine, as well as native or denatured, reduced forms of proteins tagged with 6× histidine, source: mouse, Cat. H1029-.2ML, Lot 121M4789) to detect 6×His-tagged DR5 cytoplasmic region or its mutants. Following overnight antibody incubation, membranes were washed three times with TBST, and then a 1-h incubation with horseradish peroxidase-conjugated secondary antibodies from Southern Biotechnologies (Birmingham, Alabama, specificity: anti-rabbit IgG (H+L) source: goat Cat. 405-05, Lot K0008-VJ213 or specificity: anti-mouse IgG (H+L), source: goat, Cat. 1031-05, Lot H2710-NA02) at room temperature. Proteins were evaluated using enhanced chemiluminescence detection reagents from Thermo Fisher Scientific according to the manufacturer's instructions.

DR5 Death Domain Structure Construction—The initial structure of DR5 death domain (DD) was constructed based on

the structures of tumor necrosis factor receptor (TNFR)-1 DD (PDB ID: 1ICH) (57) and Fas DD (PDB ID: 1DDF) (58) with MODERLLER 9.2 (59). Fas, TNFR-1 and DR5 all belong to the TNFR superfamily and their death domains perform similar functions during the formation of signaling complex for apoptosis (54, 60) The T-Coffee software that is a multiple sequence alignment program provided by European Bioinformatics Institute (EBI) was used for the sequence alignment of the target protein DR5 DD and the templates of Fas DD and TNFR-1 DD. Transitive consistency score function (61) implemented in T-Coffee software was used to evaluate protein sequence alignment results and the transitive consistency score for Fas DD and TNFR-1 DD were separately 79 and 80 at a 0–100 scale. The stereochemical quality of the top five candidates for DR5 DD structure that are constructed based on the template of Fas DD and TNFR-1 DD was assessed with PROCHECK, (62) ERRAT (63), and WHATCHECK (64) using NIH structural analysis and verification server (SAVES) (65, 66). PROCHECK evaluates the residual φ/ψ angles in the Ramachandran plots (62). ERRAT analyze patterns of non-bonded interactions and gives an overall quality score for the entire model (63). WHATCHECK does extensive checking of many stereochemical parameters of the residues in the model (64).

Electrostatic Potential Calculations—The electrostatic potentials for DR5 DD and DR5 DD mutants were calculated with APBS software (67). To calculate the electrostatic potential, dielectric constants of 1 and 78.54 were used for the protein and solvent separately. The ion concentration of 150 mM was used in APBS calculation. The electrostatic potential was mapped onto the molecular surface of DR5 DD using the VMD program (68).

Results

CaM Interactions with DR5 in a Ca^{2+}-dependent Manner in Triple-negative MDA-MB-231 and ER-positive ZR-75-1 Breast Cancer Cells—CaM may interact with target proteins via a calcium (Ca^{2+})-dependent or independent manner (45). We characterized the interaction of endogenous CaM and DR5 in a Ca^{2+}-dependent manner in ER-positive ZR-75-1 and triple-negative MDA-MB-231 breast cancer cells that are sensitive to TRA-8 (9, 70, 71).

Co-immunoprecipitation (Co-IP) of DR5 and CaM from MDA-MB-231 and ZR-75-1 Cell Lysate—DR5 was immunoprecipitated (IP) from each cell lysate using TRA-8-conjugated Sepharose beads. Mouse Iso-IgG-conjugated Sepharose beads were used for the control IP experiments. Western blot analysis of Co-IP samples, using primary antibodies against DR5 and CaM, was performed to assess DR5 association with CaM. Results show that CaM was co-immunoprecipitated with DR5 from ER-positive ZR-75-1 and triple-negative MDA-MB-231 breast cancer cell lysates (IP: TRA-8 lane) (Fig. 1*A*). CaM or DR5 was not found in the control Co-IP using the mouse Iso-IgG-conjugated Sepharose beads, which indicated that CaM or DR5 did not interact with the Sepharose bead of mouse IgG portion of the TRA-8 antibody. Co-IP results using TRA-8-conjugated Sepharose beads demonstrated that CaM interacted with DR5 in both ER-positive ZR-75-1 and triple-negative MDA-MB-231 breast cancer cells.

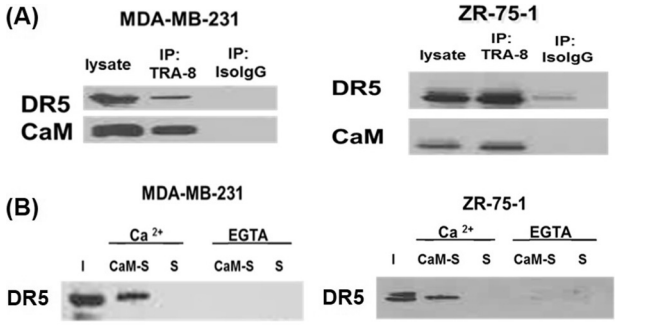

FIGURE 1. **CaM interactions with DR5 in a Ca^{2+}-dependent manner in triple-negative MDA-MB-231 and ER-positive ZR-75-1 breast cancer cells.** *A*, co-immunoprecipitation of DR5 and CaM from MDA-MB-231 and ZR-75-1 cell lysates. *Lysate lane:* whole cell lysate of breast cancer cells; *IP TRA-8 lane:* IP using TRA-8-conjugated beads; *IP Iso-IgG lane:* IP using a mouse Iso-IgG-conjugated beads. *B*, CaM pull-down of DR5 from MDA-MB-231 and ZR-75-1 cell lysates supplemented with 1 mM Ca^{2+} or 2 mM EGTA. *I*: input, *CaM-S*: pull-down using CaM-conjugated Sepharose beads. *S:* pull-down control using Sepharose beads. Representative results are shown from two independent experiments.

CaM Pull-down of DR5 from MDA-MB-231 and ZR-75-1 Cell Lysate—We characterized CaM and DR5 interactions in a Ca^{2+}-dependent manner by using CaM-conjugated Sepharose beads to pull-down DR5 from ER-positive ZR-75-1 and triple-negative MDA-MB-231 breast cancer cell lysates supplemented with 1 mM Ca^{2+} or 2 mM EGTA. Sepharose beads were used for the control pull-down experiments. Western blot analysis of pull-down samples, using primary antibody against DR5, was performed to characterize DR5 association with CaM. Results show that DR5 was present in the CaM pull-down from both types of breast cancer cell lysates supplemented with calcium (Fig. 1*B*). However, DR5 was not present in the CaM pull-down experiments from breast cancer lysates supplemented with EGTA (Fig. 1*B*). DR5 was not present in the control experiments using Sepharose beads for pull-down control experiments. The results demonstrated that CaM and DR5 interaction in ER-positive ZR-75-1 and triple-negative MDA-MB-231 breast cancer cells was calcium dependent.

CaM Directly Interacts with DR5 at DR5 Death Domain in a Ca^{2+}-dependent Manner—To determine a direct interaction between CaM and DR5, CaM pull-down assays were performed using purified recombinant 6×His-tagged DR5 cytoplasmic region (amino acids 232–440) (DR5 CR) and DR5 death domain (DD) proteins. DR5 CR or DR5 DD in 50 mM Tris, pH 7.6, 120 mM NaCl, 1% Brij buffer with 1 mM Ca^{2+}, or 2 mM EGTA were incubated with CaM-Sepharose beads or Sepharose beads. The 6×His-tagged DR5 CR or DR5 DD proteins were detected using an anti-polyhistidine antibody by Western blot. Results show that both DR5 CR and DR5 DD bound to CaM but not to the control Sepharose beads in the presence of calcium (Fig. 2*A*). However, with the medium supplemented with EGTA, either DR5 CR or DR5 DD did not bind to CaM. The results show the direct interactions between CaM and DR5 in a Ca^{2+}-dependent manner and CaM-DR5 interactions are not mediated by other DR5-binding proteins, such as FADD (20). To further confirm that CaM directly binds to DR5 at the DR5 death domain, we generated a DR5 cytoplasmic region mutant with the deletion of the DR5 death domain (DR5 CR ΔDD) and performed CaM pull-down assay of DR5 CR ΔDD

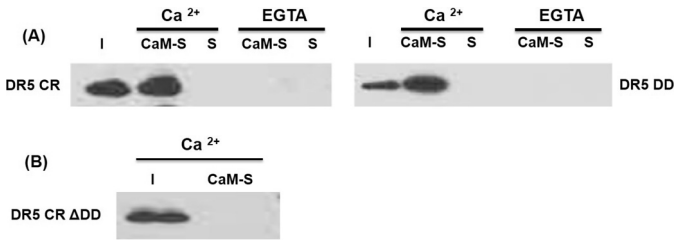

FIGURE 2. **CaM directly interacts with DR5 in DR5 death domain in a Ca^{2+}-dependent manner.** *A*, CaM pull-down of purified DR5 cytoplasmic region (DR5 CR) and DR5 death domain (DR5 DD) in a 50 mM Tris, pH 7.6, 120 mM NaCl, 1% Brij buffer with 1 mM Ca^{2+}, or 2 mM EGTA. *B*, CaM pull-down of purified DR5 CR mutant with the deletion of DR5 death domain (DR5 CR ΔDD) in a 50 mM Tris pH 7.6, 120 mM NaCl, 1 mM Ca^{2+}, 1% Brij buffer. *I*: input, *CaM-S*: pull-down using CaM-conjugated Sepharose beads. *S*: pull-down control using Sepharose beads. Representative results are shown from two independent experiments.

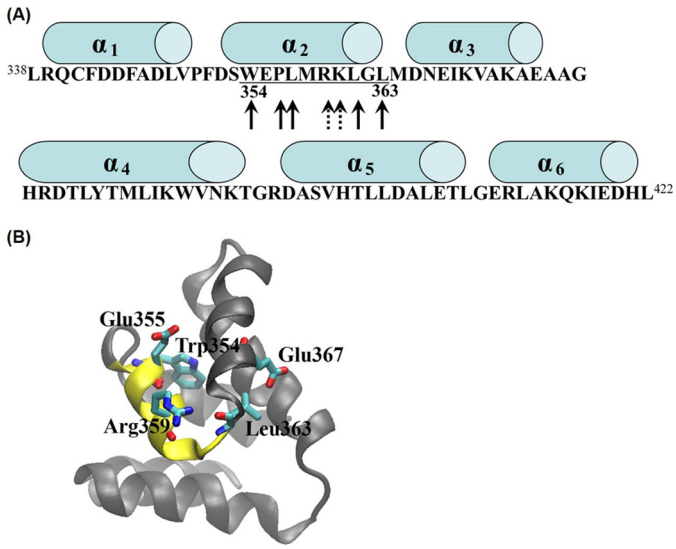

FIGURE 3. **Predicted CaM-binding site in DR5 death domain (DR5 DD) and the key residues critical for CaM-DR5 binding.** *A*, predicted CaM-binding site in DR5 DD (^{354}WEPLMRKLGL^{363}) is *underlined*. *Arrows* with *solid lines* indicate hydrophobic residues, and *arrows* with *dotted lines* indicate positively charged residues. *Cylinders* above the sequence represent α helical regions. *B*, structural view of DR5 DD with the predicted CaM-binding site (^{354}WEPLMRKLGL^{363}) shown in *yellow color* and the predicted key residues of Leu-363, Trp-354, Glu-355, Arg-359, and Glu-367 in DR5 DD shown as *licorice*.

with CaM-Sepharose beads or Sepharose beads. Results show that DR5 CR ΔDD did not bind to CaM (Fig. 2*B*). These results demonstrated that CaM directly binds to DR5 in a Ca^{2+}-dependent manner, and the interaction of CaM with DR5 was localized at the DR5 death domain.

Prediction of the CaM-binding Site on DR5 Death Domain and the Residues in DR5 Death Domain Critical for CaM-DR5 Binding—The CaM-binding site on DR5 was firstly predicted with the pattern search method (46) by scanning the three CaM-binding motifs (34, 45) separately on the sequence of DR5 DD. Five potential CaM-binding sites on DR5 DD predicted from the pattern search method are: 1) ^{338}LRQCFDDFADLVPF^{351}; 2) ^{342}FDDFADLVPFDSW^{354}; 3) ^{390}VNKTGRDASVHTLL^{403}; 4) ^{406}LETLGERLAKQKI^{418}; and 5) ^{354}WEPLMRKLGL^{363}. To further narrow down the CaM-binding site in DR5 DD, we used complementary hydropathy index sign-matching strategy (55) that is based on the molecular recognition theory of Blalock (56) to predict protein-protein interaction sites. We used the hydropathy binary code (+ and −) of the predicted CaM-binding site(s) in DR5 DD from text pattern search method to match the binary code of hydropathy of the CaM and rank the results by the number/percentage of the complementary pairs ((+)-(−)). The sequence of ^{354}WEPLMRKLGL^{363} on DR5 DD was ranked the highest as the potential CaM-binding site in DR5 DD. ^{354}WEPLMRKLGL^{363} is located at the α2 helix and the loop between α2 helix and α3 helix of DR5 DD (Fig. 3*A*).

Experimental studies have shown that the mutations of several key residues in DR5 DD including DR5 W354A, DR5 E355K, DR5 R359A, DR5 L363N, and DR5 E367K mutations result in DR5 unable to recruit FADD and caspase-8 for DISC formation to signal apoptosis (24–26, 32, 33, 44, 54). Residues of Trp-354, Arg-359, Glu-355, Leu-363, and Glu-367 are on or close to the predicted CaM-binding site in DR5 DD. Thus, we predicted that the mutations of these key residues could also affect CaM-DR5 binding, which could further affect DR5-mediated DISC formation for apoptosis. The structure of DR5 DD was constructed based on the structures of tumor necrosis factor receptor (TNFR)-1 DD (PDB ID: 1ICH) (57) and Fas DD (PDB ID: 1DDF) (58) with MODERLLER 9.2 (44, 59) and the constructed DR5 DD structure was evaluated with the NIH Structural Analysis and Verification Server as described under "Experimental Procedures." The structure with the overall best score of stereochemical quality (supplemental Table S2) was used for this study. The predicted CaM-binding site in DR5 DD and the residues in DR5 DD critical for CaM-DR5 binding were shown in the three-dimensional structure of DR5 DD (Fig. 3*B*).

Validation of the Predicted CaM-binding Site in DR5 DD and the Key Residues in DR5 DD Critical for CaM-DR5 Binding—We performed biochemical experiments to verify the predicted CaM-binding site in DR5 DD (^{354}WEPLMRKLGL^{363}) and the key residues in DR5 DD critical for CaM-DR5 binding. We generated the constructs for DR5 cytoplasmic region (DR5 CR), DR5 CR mutant with the deletion of the predicted CaM-binding site in DR5 DD (DR5 CR ΔBS), and DR5 CR mutants with the point mutation of W354A, E355K, R359A, L363N, or E367K. Residues of Trp-354, Arg-359, Glu-355, Leu-363, and Glu-367 are on or close to the predicted CaM-binding site in DR5 DD. We expressed and purified DR5 CR protein and DR5 CR mutants as described under "Experimental Procedures." We performed pull-down experiments for the purified recombinant DR5 CR, DR5 CR ΔBS mutant, and the mutants of DR5 CR W354A, DR5 CR E355K, DR5 CR R359A, DR5 CR L363N, and DR5 CR E367K separately using CaM-Sepharose beads. Results show that the deletion of the predicted CaM-binding site (^{354}WEPLMRKLGL^{363}) in DR5 cytoplasmic region resulted in the loss of the CaM-DR5 binding (Fig. 4*A*). The point mutation of W354A, E355K, R359A, L363N, or E367K in DR5 CR resulted in the decreased CaM-DR5 binding compared with CaM binding to wild type DR5 CR (Fig. 4*B*). These results show that the predicted CaM-binding site in DR5 DD is required for CaM-DR5 binding, validating the predicted CaM-binding site in DR5: ^{354}WEPLMRKLGL^{363}. The results also demonstrate that the point mutations of the residues of Trp-354, Arg-359, Glu-355, Leu-363, and Glu-367 that are on or close to the pre-

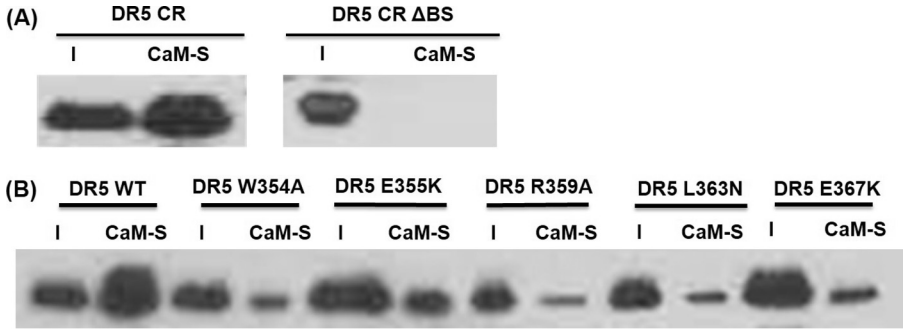

FIGURE 4. **Validation of the predicted CaM-binding site in DR5 DD and the effect of critical residues in DR5 DD on CaM-DR5 binding.** *A*, CaM pull-down of DR5 cytoplasmic region (DR5 CR) or DR5 CR mutant with the deletion of the predicted CaM-binding site (^{354}WEPLMRKLGL^{363}) (DR5 CR ΔBSD). *B*, CaM pull-down of wild type DR5 CR (WT) or DR5 CR mutants: DR5 W354A, DR5 E355K, DR5 R359A, DR5 L363N, and DR5 E367K. *I*: input, *CaM-S*: pull-down using CaM-conjugated Sepharose beads. Representative results are shown from two independent experiments.

dicted CaM-binding site in DR5 death domain directly affect CaM-DR5 binding. These residues have been shown to be important for DR5 recruitment of FADD and caspase-8 for DISC formation to signal apoptosis (24–26). These results suggest the important role of CaM-DR5 binding in DR5-mediated DISC formation for apoptosis.

Electrostatic Potential Changes in DR5 DD Resulted from the Point Mutation of the Key Residues in CaM-binding Site in DR5 DD—The electrostatic potential changes of the CaM-binding site in DR5 DD could directly affect CaM-DR5 binding. To understand the effect of the point mutations of the key residues in DR5 DD, including W354A, E355K, R359A, L363N, or E367K mutation on CaM-DR5 binding, we performed electrostatic potential analyses for DR5 DR5 DD WT and DR5 DD mutants using APBS software (67). The comparisons of the electrostatic potential of the CaM-binding site in DR5 DD WT and in DR5 DD mutants are shown in Fig. 5, in which the blue color represents the positive electrostatic potential whereas the red color represents the negative electrostatic potential. The results show that for DR5 DD WT, the positive electrostatic potential was distributed in the middle region of the CaM-binding site, whereas the negative electrostatic potential was distributed in the upper and lower regions of the CaM-binding site (Fig. 5). For DR5 DD W354A mutant, the positive electrostatic potential distribution in the middle region of the CaM-binding site in DR5 DD was much smaller compared with that in DR5 DD WT, and the negative electrostatic potential was only distributed in the lower region (Fig. 5*A*). For the DR5 DD E355K mutant, the positive electrostatic potential in the middle region of the CaM-binding site in DR5 DD was significantly increased compared with that in DR5 DD WT and the negative electrostatic potential was only distributed in the left upper region of the CaM-binding site in DR5 DD (Fig. 5*B*). For the DR5 DD R359A mutant, the positive electrostatic potential only appeared in a small region of the middle of the CaM-binding site in DR5 DD and the negative electrostatic potential was significantly increased in the left top and bottom region (Fig. 5*C*). For the DR5 DD E367K mutant, although Glu367 is not on the CaM-binding site in DR5 DD, the E367K mutation also affects electrostatic potential distributions in the CaM-binding site in DR5 DD, resulting in the increased positive electrostatic potential distribution in the top half region of the CaM-binding site in DR5 DD (Fig. 5*D*). For the DR5 DD L363N mutant, although the electrostatic potential pattern was similar to that in DR5 DD WT, however, the degree/magnitude of electrostatic potential distribution resulting from the L363N mutation was different from that for DR5 DD WT (Fig. 5*E*). The electrostatic potential changes resulting from the key residue mutations in DR5 DD could directly affect CaM-DR5 interactions, further affecting the degree of CaM-DR5 binding.

Discussion

DR5 is one of the well characterized death receptors, and DR5 contains a cytoplasmic death domain (12) and similar to the Fas receptor transduces its apoptotic signal via DISC formation and activation of caspase signaling for apoptosis (18, 20). Moreover, not only mediating apoptosis, DR5 also mediate anti-apoptotic signaling in breast cancer cells (69). These suggest that DR5 engage a variety of biological effect. So far, significant studies about the downstream molecules related to DR5-mediated apoptotic and anti-apoptotic functions have been reported, including FADD, glycogen synthase kinase-3 DDX3 and cellular inhibitor of apoptosis protein-1 (18, 69). DR5 is expressed in various breast cancer cell lines that represent the different breast cancer subtypes (9, 10), and DR5 expression is up-regulated in breast cancer cells compared with normal breast tissue (11). Although the studies about DR5-mediated apoptotic signaling and anti-apoptotic signaling in breast cancer cells has been reported (9, 69). However, many aspects of DR5-mediated biological effects remain unknown, which could be mediated by the interactions of DR5 with various other molecules.

In this study, we characterized the interactions between CaM and DR5 in ER-positive ZR-75-1 and triple-negative MDA-MB-231 breast cancer cells with integrated experimental and computational approaches. The results show that CaM directly binds to DR5 in a calcium-dependent manner in both ER-positive ZR-75-1 and triple-negative MDA-MB-231 breast cancer cells. Results also show that the direct interaction of CaM with DR5 was localized at the DR5 death domain. We have predicted and verified the CaM-binding site in DR5 being ^{354}WEPLMRKLGL^{363} that is located at the α2 helix and the loop between α2 helix and α3 helix of DR5 DD. These findings are important not only for understanding the DR5-mediated apoptosis but also important for expanding the knowledge of CaM. CaM functions as an intracellular mediator of Ca^{2+}

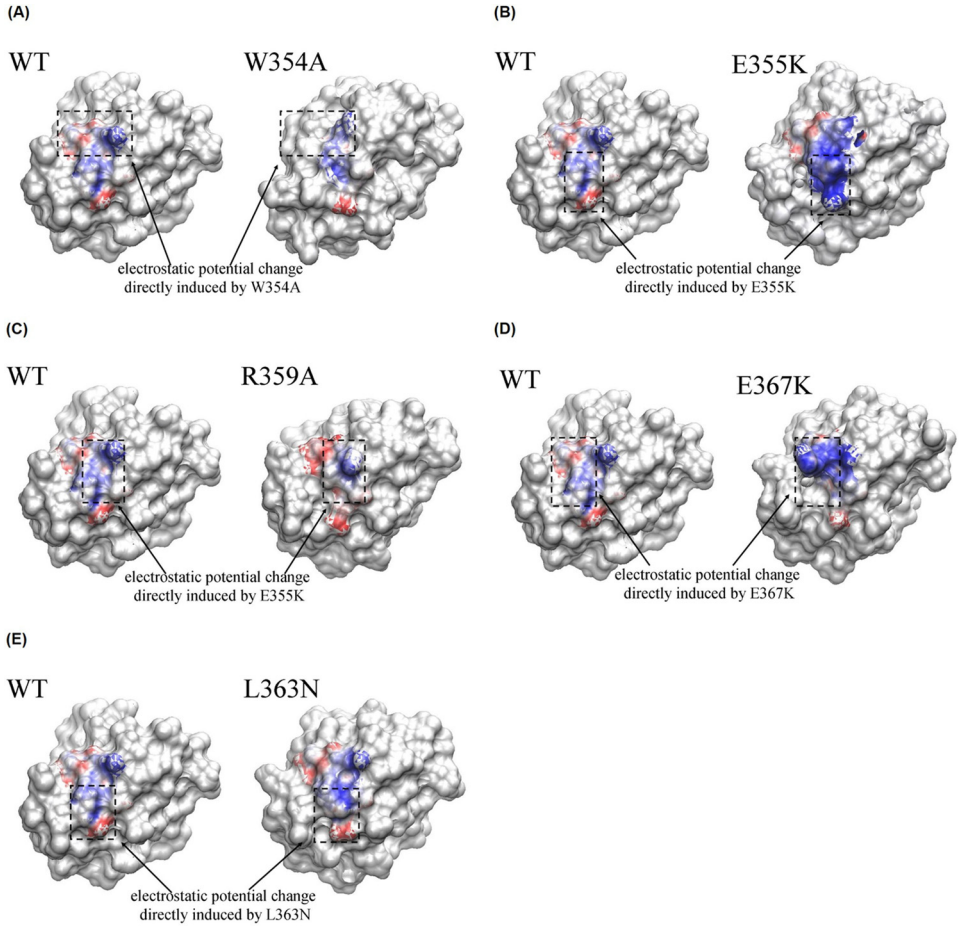

FIGURE 5. **Electrostatic potential comparison between the CaM-binding site in wild type DR5 DD and DR5 DD mutants.** The *blue regions* show the positive electrostatic potential, whereas the *red regions* show the negative electrostatic potential. The *black dot boxes* show the electrostatic potential comparison *between* the CaM-binding site in wild type DR5 DD and DR5 DD mutants (*A*) W354A mutant, (*B*) E355K mutant, (*C*) R359A mutant, (*D*) E367K mutant, and (*E*) L363N mutant.

signals and regulates various cellular processes (29, 30). Breast tumor transformation to malignancy is associated with the increase in CaM expression (31, 32). CaM binding to Fas has been well characterized (33–37). Results from this provide the potential mechanism for CaM regulation of DR5-mediated apoptotic signaling in breast cancer.

The residues of Trp-354, Arg-359, Glu-355, Leu-363, and Glu-367 in the DR5 death domain have been shown important for DR5 recruitment of FADD and caspase-8 for DISC formation to signal apoptosis (24–26). Results from this study show that the point mutations of W354A, E355K, R359A, L363N, or E367K in the DR5 death domain directly affect CaM-DR5 binding, which suggest the important role of CaM-DR5 binding in DR5-mediated DISC formation for apoptosis. Our electrostatic potential analysis results show that the mutations of W354A, E355K, R359A, L363N, or E367K in DR5 DD directly affect the electrostatic potential distribution in the CaM-binding site in DR5 DD, which help to interpret our experimentally observed decreased CaM-DR5 binding by the point mutation of these key residues in DR5 DD. The results from this study provide the basis for the further investigation of the role of CaM-DR5 binding in DR5-mediated DISC formation for apoptosis in ER-positive and triple-negative breast cancer cells.

Author Contributions—R. M. F., Y. S., and T. Z. designed the research. R. M. F., Q. Z., and H. W. conducted the biochemical experiments and analyzed experimental data. L. W. conducted the computational studies. L. W. and Y. S. analyzed computational data. T. Z. and D. J. B. provided guidance for biochemical experiments. R. M. F., L. W., and Y. S. wrote the manuscript. Q. Z., H. W., T. Z. D. J. B. provide inputs and assistance for the manuscript writing. All authors reviewed the results and approved the final version of the manuscript.

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